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FORM PTO-1390 (REV 10-96)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 599-158P
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>08/809621</b>
INTERNATIONAL APPLICATION NO. PCT/JP96/02099	INTERNATIONAL FILING DATE 25 July 1996	PRIORITY DATE CLAIMED 25 July 1995	
TITLE OF INVENTION REMEDY FOR BONE DISORDERS			
APPLICANT(S) FOR DO/EO/US IDA, Nobutaka; SUZUKI, Tomohiko; KUMAGAI, Emi			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li>4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</li> <li>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol>			
Items 11. to 16. below concern document(s) or information included:			
<ol style="list-style-type: none"> <li>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li>13. <input type="checkbox"/> A <b>FIRST</b> preliminary amendment. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</li> <li>14. <input type="checkbox"/> A substitute specification.</li> <li>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li>16. <input checked="" type="checkbox"/> Other items or information:             <ul style="list-style-type: none"> <li>✓ International Search Report (PCT/ISA/210)</li> <li>✓ Ten (10) sheets of formal drawings</li> </ul> </li> </ol>			

NEW

PCT/JP96/02099

599-158P

17. ☒ The following fees are submitted:**BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):**

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Total claims	10 - 20 =	0	X \$22.00
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overpayment to Deposit Account No. 02-2448. A duplicate copy of this sheet is enclosed.**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR  
1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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28,977

REGISTRATION NUMBER

/djm 03/25/97

SPECIFICATION

Drug For Treating Bone Disorders

TECHNICAL FIELD

5 The present invention relates to use of interferon  $\beta$   
as a drug for bone disorders. The drug is used for  
preventing or curing bone disorders caused by various  
causes such as physical and metabolic causes and by  
malignant tumors. The present invention also includes  
10 use of interferon inducers as a drug for preventing and  
curing bone disorders.

BACKGROUND ART

Bones are important tissues in vertebrates, which  
support the body. Unlike the image from their outer  
appearances, bones actively repeat degradation of bones  
15 by osteoclasts and formation of bones by osteoblasts,  
thereby attaining the homeostasis of their morphology and  
physical strength based on the exquisite balance  
therebetween. If this balance is lost, various bone  
disorders are caused. In recent years, osteoporosis is  
20 especially a big medical and social problem (H. Orimo and  
H. Ozawa, "Graphically Shown Osteoporosis", MEDICAL VIEW,  
1990). Osteoporosis does not necessarily occur when the  
osteoclastic activity is abnormally high, but also occurs,  
for example, even when the osteoclastic activity is low  
25 if the bone formation is lower. Osteoporosis includes  
the high turnover type in which both the bone resorption  
and bone formation are active, and the low turnover type

in which both the bone resorption and bone formation are inactive. The former is also called type I and frequently occurs among postmenopausal women, and the latter is also called type II and frequently occurs among old people irrespective of sex. Thus, what is important is the relative balance between the bone formation and the osteoclastic activity, and the balance is finally reflected to the bone volume and bone strength.

Therefore, it is thought effective for therapy of bone disorders accompanying decrease in bone mineral density to relatively increase the rate of bone formation with respect to the rate of bone resorption or to relatively decrease the rate of bone resorption with respect to the rate of bone formation.

In the bone resorption process, osteoclasts play the most important role (Baron, R. et al., Bone and Mineral Res., 2, 175-243, Elsevier, New York, 1984). In addition to physiological causes such as menopause and aging, bone disorders are caused by various physical, chemical and biological causes as well as by unknown causes, which influence on the proliferation, differentiation or expression of functions of these cells so as to disturb the relative balance between the bone resorption and bone formation. The disorders in which the relative balance between the bone resorption and bone formation is disturbed include cancer-related diseases such as bone metastasis of lung cancer, mammary carcinoma or renal

cancer, and multiple myeloma; metabolic bone disorders such as Paget's disease, rickets, osteomalacia, marble bone disease (osteopetrosis), osteoarthritis and osteogenesis imperfecta; diseases related to hormonal disorders, such as Cushing's syndrome and hyperparathyroidism; autoimmune disorders such as rheumatoid arthritis; and periodontal diseases such as alveolar bone disorder. Osteoblasts are mononucleated cells differentiated from immature mesenchymal cells originated from mesoderm. Since osteoblasts separated from bone tissues of experimental animals have the property to divide and proliferate *in vitro*, a number of cell lines have been established. Examples of such established cell lines include MC3T3-E1 (Sudo, H. et al., J. Cell Biol., 96, 191, 1983), ROS17/2, UMR106 (Fraser, J.D. et al., J. Biol. Chem., 263, 911, 1988) and RCT-3 (Hearth, J. K. et al., Endocrinology, 124, 3060, 1988).

Osteoclasts are multinucleated giant cells (Wergedal, J. E., Proc. Soc. Exp. Biol. Med., 134, 244, 1970, Clark, S.E. et al., J. Bone Mineral Res., 4, 399, 1989), having the features that they have calcitonin receptors (Warshawsky, H. D. et al., J. Cell Biol., 85, 682, 1980, Nicholson, G. C. et al., J. Clin. Invest., 78, 355, 1986), and that in bone resorption, they ionize the calcium contained in the bone matrix and transport the calcium to body fluid (Blair, H.C. et al., Science, 245, 855, 1989). Embryologically, osteoclasts are formed by

differentiation of bone marrow cells. It is known that osteoclast like cells which express the fundamental properties of osteoclasts are formed by culturing mononuclear cells of bone marrow or spleen in the presence of 1,25-dihydroxyvitamin D<sub>3</sub> or prostaglandin E<sub>2</sub> (Takahashi, N. et al., Endocrinology, 122, 1373, 1988, Udagawa, N. et al., Endocrinology, 125, 1805, 1989).

Interferon (hereinafter referred to as "IFN" for short) is a protein which exhibits various physiological activities including antiviral and antitumor activities. Interferon is now classified into  $\alpha$ ,  $\beta$  and  $\gamma$  types depending on the differences in history of discovery, structure and physicochemical properties. For example, IFN $\alpha$  is mainly produced by leukocytes, lymphoblastoid cells, NK cells, B cells and the like; IFN $\beta$  is mainly produced by fibroblast cells; and IFN $\gamma$  is mainly produced by T cells and NK cells. All of these types of IFN are now commercially available as an antitumor agent or an agent for treating hepatitis and clinically used for human. Since administration of 1,25-dihydroxyvitamin D<sub>3</sub> or calcitonin to patients suffering from osteoporosis increases the blood level of IFN $\alpha$  (Shiozawa, S. et al., Gerontol., 35, 305, 1989), some relationship was suggested (T. Fujita, Clinical Calcium, 2, 852, 1992). Inhibition of formation of osteoclast like cells by various recombinant IFN $\alpha$  *in vitro* in human cord blood cells has been reported (K. Anai et al., Nihon-

Kotsutaisha-Gakkai Zassi (in Japanese) Vol. 9, 238, 1991).

Further, it has been reported that administration of human recombinant IFN $\alpha$  to patients suffering from hepatitis C decreases the level of urinary

5 deoxypyridinoline which is an index of the osteoclastic activity (H. Yoshida et al., Nihon-Kotsutaisha-Gakkai Zassi, Vol. 12, 215, 1994) or increases the bone volume (M. Kawakatsu et al., Nihon-Kotsutaisha-Gakkai Zassi, Vol. 12, 234, 1994). On the other hand, however, it has also  
10 been reported that administration of human recombinant IFN $\alpha$  to patients suffering from hepatitis C does not influence on the bone volume of the patients at all (T. Tanaka et al., Japanese Journal of Gastroenterology, Vol. 91, Subject 0-423, 1994). Thus, evaluation of IFN $\alpha$  is in  
15 chaos. The chaotic state about the existence of the effects is also seen in the basic studies. That is, it has been reported that murine IFN inhibits bone resorption *in vitro* induced by parathyroid hormone in an organ culture system of bone (Jilka, R. L. and Hamilton, J. W., Biochem. Biophys. Res. Commun., 120, 553, 1984).  
20 On the other hand, it has also been reported that administration of murine IFN to mice in which decalcified bone matrix was subcutaneously transplanted gives no effects at all *in vivo* (Nilsson, O.S. et al., J. Interferon Res., 4, 135, 1984). The IFN used in both of  
25 these experiments was a mixture of IFN $\alpha$  and  $\beta$ . Further, it has been reported that IFN $\alpha$  is not effective against

rheumatoid arthritis at all (Kajandahl, A. et al., Lancet, Vol. i, 984, 1979) and even there is a report which insists the disadvantage of IFN, that is, which reports that IFN induces experimental synovitis and that IFN induces autoimmune diseases in human (Rosenbach, T.O. et al., Clin. Rheumatol., 3, 361-364, 1984). Thus, the evaluation of IFN *per se* has not been fixed and the existence of effects of IFN $\beta$  alone is not known at all.

The substantial part of the reason why it is unclear whether an effect of IFN on bone metabolism exists or not is the species specificity of IFN. Especially, unlike IFN $\alpha$ , IFN $\beta$  has a strong species specificity. Therefore, evaluation of the effect of IFN $\beta$  must be made using the IFN $\beta$  of the animal of the species of interest. As mentioned above, increase and decrease in bone volume is finally determined by the relative strength of the effect on the bone formation system and on the bone resorption system, the correct evaluation of IFN $\beta$  is attained only by a comparative experiment on the strictly and scientifically controlled bone resorption system and bone formation system using the IFN $\beta$  from the animal species of interest. The present inventors insist that it is indispensable to the development of a drug for bone disorders to make relative evaluations on both bone formation system and bone resorption system. The correctness of this opinion will be concretely shown by the example employing IFN $\gamma$  described below.



The reports about IFN $\gamma$  are most abundant among the reports about the influences by IFN on bone metabolism. Most of the reports utilize the inhibition of formation of osteoclast like cells from bone marrow cells in the experimental system established by Takahashi et al., which inhibition is attained by stimulation by bradykinin (Lerner, U.H. et al., Agents and Actions, 32, 305, 1991), forskolin, cholera toxin (Lerner, U.H. et al., J. Bone Min. Res., 6, 551, 1991), interleukin 1 (Foffmann, O. et al., Biochem. Biophys. Res. Commun., 143, 38, 1987) or by 1,25-dihydroxyvitamin D<sub>3</sub> (Gowen, M. et al., J. Bone Min. Res., 1, 469, 1986). Prior to these reports, the action of IFN $\gamma$  on bone metabolism had already been disclosed in a patent application (M. Peterlik, Japanese Patent Application No. 61-123862). If evaluation is made based on the position that evaluation in the bone resorption system alone is sufficient, inhibition of formation of osteoclasts should reduce or stop the decrease in bone volume, or increase the bone volume since osteoclasts carry out bone resorption, so that the invention directed to the use of IFN $\gamma$  as a drug for improving bone volume may be regarded as completed or may be inferred easily. However, the truth was on the contrary. It has been reported that administration of IFN $\gamma$  has no therapeutic effects against osteopenia (according to Stedman's Medical Dictionary, Revised 2nd Edition, Medical View Co., Ltd., 1988, osteopenia means deposition of calcium or

decrease in density of bone), but decreases the bone volume, and it was suggested that the reason therefor may be the inhibition of deposition of bone mineral (Mann, G. N. et al., Endocrinology, 135, 1077, 1994). Further, 5 ironically, the later studies revealed that IFN $\gamma$  having strong osteoclast formation-inhibition activity exhibited excellent therapeutic effect against marble bone disease against which promotion or activation of bone resorption by formation of osteoclasts is needed for therapy or 10 relief of this disease in both an animal (Rodriguez, R.M. et al., Pediatrics Res., 33, 384, 1993) and in human (Lyndon Key, Jr. L. et al., NewEngl. J. Med., 332, 1544, 1995).

Thus, in order to be a drug effective for therapy of 15 bone disorders, especially the bone disorders requiring increase in the bone volume, the fact that the drug or the substance exhibits inhibition of bone resorption is insufficient, but the fact that the drug or the substance does not inhibit at all or only slightly inhibits the 20 bone formation system (proliferation, differentiation and mineralization of osteoblasts), or preferably, the fact that the drug activates the bone formation system, must be proved. In fact, by solving the problem described below, the present inventors reached a conclusion which 25 is contrary to the above-mentioned observation by Nilsson et al. (J. Interferon Res., 4, 135, 1984) that administration of mouse IFN is not effective at all for

bone formation in mice. That is, the present inventors completed the present invention by proving the effect for promoting bone formation so as to reinstate the bone volume which was once decreased in bone disorder model animals.

#### DISCLOSURE OF THE INVENTION

According to the present invention, based on the relative evaluation of both the strictly controlled bone resorption system and bone formation system using IFN $\beta$  free from the ambiguity of the substance, the use of IFN $\beta$  as a drug for preventing and treating bone disorders, which is useful in industry and in medicine is provided. Further, based on the discoveries during the process of completing the present invention, use of IFN $\alpha$ ,  $\beta$  and  $\gamma$  inducers as a drug for preventing and treating bone disorders is provided by the present invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows inhibition actions of murine IFN $\beta$  and  $\gamma$  against the formation of osteoclast like cells. The bone marrow cells are originated from mice of ddy strain.

Fig. 2 shows inhibition actions of murine IFN $\beta$  and  $\gamma$  against the formation of osteoclast like cells. The bone marrow cells are originated from mice of C 57 BL/6 strain.

Fig. 3 shows actions of murine IFN $\beta$  and  $\gamma$  on the proliferation of murine osteoblastic cells MC3T3-E1.

Fig. 4 shows the difference between the actions by murine IFN $\beta$  and  $\gamma$  on the mineralization of murine

osteoblastic cells MC3T3-E1.

Fig. 5 shows the amount of calcium extracted from the calcified foci of murine osteoblastic cells MC3T3-E1 treated with murine IFN.

5 Fig. 6 shows the amount of inorganic phosphorus extracted from the calcified foci of murine osteoblastic cells MC3T3-E1 treated with murine IFN.

Fig. 7 shows effect for promoting mineralization in murine osteoblastic cells MC3T3-E1 by frequent  
10 administration of murine IFN $\beta$ .

Fig. 8 shows the effect for restoring bone volume of mice by administration of IFN $\beta$ , which bone volume was decreased by ovariectomy.

Fig. 9 shows inhibition effect by administration of  
15 murine IFN $\beta$  on the bone resorption which was increased by ovariectomy.

Fig. 10 shows change in body weights of mice during the experiment of administration of murine IFN $\beta$ .

#### BEST MODE FOR CARRYING OUT THE INVENTION

20 To eliminate the ambiguity of the substance, the present inventors used highly purified murine IFN. For this purpose, large scale production by genetic engineering technique followed by purification of the product is one of good ways. Details of this method have  
25 been reported (Murine IFN $\alpha$ : Shaw, G.D. et al., Nucleic Acids Research, 11, 556-573, 1982; Murine IFN $\beta$ : Higashi, Y. et al., J. Biol. Chem., 258, 9522, 1983, Senda, T. et

al., Proc. Japan Acad., 66, Ser.B,77, 1990; Murine IFN $\gamma$ :  
Gray, P.W. and Goeddel, D.V., Proc. Natl. Acad. Sci., 80,  
5842, 1983). Needless to say, naturally occurring murine  
IFN may be used without any problem if it is sufficiently  
5 purified. In the examples of the present invention, as  
the murine IFN $\alpha$ , a commercially available product (Lot.  
B13010, 10<sup>6</sup> units/ml/vial, Calbiochem-Novabiochem  
International, San Diego, CA) was used. As the murine  
IFN $\gamma$ , a standard product Lot 254F3, 2.06 x 10<sup>6</sup> units/ml,  
10 provided by Genentech, San Francisco, CA was used. As  
the murine IFN $\beta$ , a product Lot M-0034, 10<sup>7</sup> units/ml/vial,  
prepared by Toray Industries, Inc. was used. The process  
for producing the murine IFN $\beta$  by genetic engineering  
technique as well as the method for purifying the  
15 obtained product have been reported in detail (Tanaka, T.  
et al., J. Interferon Res., 6, 429, 1986, Matsuda, S. et  
al., J. Interferon Res., 6, 519, 1986). Alternatively,  
since highly purified murine IFNs are also commercially  
available from other manufacturers (e.g., Hycult  
20 biotechnology, Paesel GmbH, Cosmo Bio and Genzyme), these  
may also be employed.

To eliminate the ambiguity in biological evaluations,  
the relative activities to the cells in the bone  
formation system and the bone resorption system from the  
25 same species, especially from the same strain, were  
quantitatively dealt with, thereby overcoming the  
drawback in the prior art. That is, even if experimental

animals of the same species are used, the response to various hormones largely differ from strain to strain, so that it is necessary to examine the relative effectiveness on the bone resorption system-bone formation system using the same strain. For example, it is known that the rates of decrease in bone volume by ovariectomy in mice which are the model of type I osteoporosis are clearly different depending on the strain (T. Hosaka, ANITEX, Vol. 5, 243, 1992). Thus, the present inventors strictly and quantitatively compared the actions on the osteoblasts responsible for the bone formation system and on the osteoclasts responsible for the bone resorption system using highly purified murine IFN, the osteoblasts and the osteoclasts being originated from the same murine strain.

As a result, IFN $\beta$  very strongly inhibits formation of osteoclasts. On the other hand, IFN $\beta$  does not substantially inhibit any of the proliferation, differentiation and mineralization of osteoblasts, and IFN $\beta$  even promotes the mineralization depending on the conditions. The present inventors revealed these facts for the first time, thereby completing the present invention. The present inventors confirmed the strong inhibition of formation of osteoclasts by IFN $\gamma$ . However, in addition, the present inventors revealed for the first time that IFN $\gamma$  strongly inhibits mineralization in the bone formation system in the simple *in vitro* system

including osteoblasts alone. This result well explains the above-mentioned result of osteoid formation in mineralization disorder *in vitro* by IFN $\gamma$  (Mann, G.N. et al., Endocrinology, 135, 1077, 1994). Mineralization is an indispensable process to the completion of bone tissues which are hard tissues. Thus, the present inventors proved that the property of IFN $\beta$  is much more preferred to that of IFN $\gamma$  for the improvement of bone volume, thereby completing the present invention.

Needless to say, the present inventors know that the strong inhibition by IFN $\beta$  against the formation of osteoclasts can be effectively utilized for prevention and treatment of bone disorders related to cancer, such as bone metastasis of, for example, mammary carcinoma, lung cancer, prostate cancer, thyroid gland carcinoma, renal cancer, colon cancer, cancer of digestive tract, cancer of esophagus and the like (Stoll, B.A., Bone Metastasis: Monitoring and Treatment, Raven Press, N.Y., 1983). This is because that the formation and development of bone metastatic foci of tumors are intimately related to increase and activation of osteoclasts (Galasco, C.B.S., Skeletal Metastases, Butterworths, pp.22-51, 1986). Therefore, needless to say, IFN $\beta$  may be effectively used for therapy of hypercalcemia (a mode of tumor-related bone disorders) accompanied by tumors. The fact that IFN strongly inhibits formation of osteoclasts *in vitro* and, on the

other hand, it does not hinder the proliferation, differentiation and mineralization of the cells of the bone formation system or even promotes the mineralization depending on the conditions, and the fact that IFN

5 inhibits *in vivo* the bone resorption and increase the bone volume which is once decreased in osteoporosis model animals clearly show that the relative evaluations in both the bone resorption system and the bone formation system are indispensable. No matter how strong the inhibition by a substance against the bone resorption system may be, this alone does not promise that the substance increases the bone volume. This is apparent from the experiments using IFN $\gamma$  described here and from the reported results (Mann, G.N. et al., Endocrinology, 10 135, 1077, 1994). That is, even if the bone resorption-inhibition action by IFN is discovered, unless the action on the bone formation system is not clarified, the invention directed to the use of IFN as a drug for preventing or treating bone disorders is incomplete.

20 The ambiguity was first overcome by the *in vitro* test in the present invention, and the invention was completed by the proof in the *in vivo* test. Further, IFN $\beta$  puts the body forward to relative bone formation, and this action is more prominently exhibited in the body in which the bone volume is abnormal (e.g., 25 ovariectomized mice which are post menopausal osteoporosis models employed in the examples of the



present invention) than in the body having the normal bone volume. That is, as described in the examples of the present invention, administration of IFN $\beta$  at a dose of  $10^3$  units/head/time to mice which became osteoporosis by ovariectomy exhibited prevention effect against further loss in the bone volume. On the other hand, although the action (bone resorption-inhibition action) inhibiting excretion of urinary deoxypyridinoline (hereinafter referred to as "D-Pyr" for short) is exhibited also in the sham operation groups, this did not immediately bring about sharp increase in the bone volume of the mice. This may be because of the homeostasis which prevents abnormal increase in the bone volume, thereby adjusting the bone volume not to reach the state such as marble bone disease. In fact, the dry bone weight was about the same as that of the control group to which physiological saline was administered. Thus, it was proved that the effect by IFN $\beta$  to increase the bone volume is more effectively exhibited in the individuals having abnormally low bone volume (due to disorders or disturbed homeostasis) than in the individuals having the normal bone volume.

These facts clearly indicate that IFN $\beta$  is very effective for prevention and therapy of bone disorders which show abnormal bone volume in the whole or a part of the bone tissues, such as metabolic bone disorders, hormonal bone disorders, osteoporosis, bone fracture,

alveolar bone disorders. In view of the effect discovered by the present invention, it is apparent that IFN can be used for bone disorders of animals other than human. However, in view of the species specificity of IFN, it is preferred to use the IFN originated from the same species as the animal to be treated. In recent years, the environments for raising companion animals such as dogs and cats have been very much improved. That is, highly advanced medical technologies are applied to the animals and feeds containing well-balanced nutrients are prevailing. As a result, the ages of the companion animals are now rapidly getting higher and higher. The various bone disorders including those in the dental field of the aged animals are surprisingly similar to those of human. Thus, the IFNs from various animals may be used for prevention and treatment of metabolic bone disorders, hormonal bone disorders, osteoporosis, bone fracture, alveolar bone disorders of the corresponding animals.

The IFN used in the present invention may be IFN $\beta$ , or consensus type or hybrid type containing the amino acid sequence of IFN $\beta$ , and may be one naturally occurring, produced by genetic engineering or by chemical synthesis.

For the production of naturally occurring IFN $\beta$ , fibroblasts as well as established cell lines thereof are preferably employed. In cases of producing IFN by genetic engineering technique, as the host cells,

mammalian cells such as CHO (Chinese hamster ovary) cells and mouse C127 cells; cells of insects such as silkworm and Barathra; cells of microorganisms such as *E. coli*, *B. subtilis* and yeasts may be employed. Further, mouse, rat, hamster, rabbit, goat, ovine, swine, bovine and the like may be employed.

The thus prepared IFN $\beta$  may be purified by applying the culture supernatant of the cells, insect extract, bacterium extract or body extract to various chromatography. Any chromatography may be employed as long as it has an affinity to IFN $\beta$ . For example, columns containing silica or calcium phosphate as an adsorbent; columns having heparin, dye or a hydrophobe as a ligand; metal chelating columns; ion-exchange columns; and gel permeation columns may be employed.

The present invention provides a novel method for search and evaluation of drugs for treating bone disorders. That is, in the conventional method, a test substance is added *in vitro* to osteoblastic cells or osteoclasts (osteoclast-forming cells) and the bone formation ability or bone resorption ability is evaluated. Similarly, in the conventional *in vivo* test, a test substance is administered to animals and bone volumes are measured. Although these methods are direct methods, the methods are time-consuming and costly when a number of test substances are tested. On the other hand, according to the method of the present invention for the primary

evaluation whether a test substance may be used as a drug for treating bone disorders or not, the IFN-producing ability of the test substance is checked *in vitro* and then *in vivo*, so that the method is simple. Further, from methodological viewpoint, the method of the present invention is an indirect method, which is distinguished from the conventional direct methods. The criteria for evaluation is firstly the absolute amount of IFN produced, and secondly the relative ratio of the IFN produced to other cytokines and lymphokines exhibiting bone resorption-promoting activities (e.g., interleukin 1, interleukin 6 and the like) produced. That is, the present inventors insist the importance of the concept of relative activity also in the search for a drug for bone disorders targeting IFN inducers. The concrete values of the above-mentioned first and second criteria for the selection of the candidate substances vary depending on the test system employed in the secondary evaluation. Any known cells may be employed for the evaluation of IFN-producing ability *in vitro*, and cells of bone tissue origin are also preferably used. The produced IFN as well as other cytokines and lymphokines may be measured by any of known methods such as bioassay, enzyme immunoassay, radioimmunoassay and the like.

25       The IFN obtained according to the present invention may be used as a drug as it is or the IFN may be admixed with one or more pharmaceutically acceptable carrier or

additive. Examples of these include almost all of the carriers suited for parenteral administration, such as water, physiological saline, Ringer's solution, Hanks' solution and glucose solution, as well as solutions of lactose, dextrose, ethanol, glycerol, albumin and the like. These compositions may contain other additives such as stabilizers, antioxidants, antibacterial agents, antiseptics, buffer agents, surfactants and the like as required. Further, to prevent or relief of flu like symptom, an anti-inflammatory drug may be incorporated in the IFN formulation. The additive or combination of additives may be appropriately selected from those described above depending on the particular formulation of the drug, although, needless to say, the additives are not restricted to those described above.

The administration route is not restricted, and appropriately selected depending on the disorder to be treated. The drug may be administered orally; parenterally or systemically, that is, subcutaneously, intramuscularly, intraperitoneally, intravaginally, intrarectally, or by intravenous injection; and/or intranasally and/or intrapulmonally. Further, a sustained release system of indwelling type such as an osmotic pump (e.g., alzet osmotic pump, Alzet Corporation, Palo Alto, CA) may be selected depending on the disorder to be treated.

When the drug is administered intranasally or

intrapulmonally, the drug may be in the form of solution or powder formulated by a medically acceptable device which produces fine solution drops or fine powder.

Further, the IFN according to the present invention may  
5 be formulated as one for topical application. For example, the drug may be directly infused to the site to be treated, or a carrier of the drug which allows for sustained release of the drug may be directly embedded in the site to be treated. Examples of such a carrier  
10 include hydrogels, polylactic acid and collagen matrices. In cases where a collagen matrix originated from an animal other than human is employed, it is preferred to use atelocollagen (such as Zyderm, Collagen Corp., Palo Alto CA) in which the antigenecity of collagen is deleted.  
15 Further, hydroxyapatite calcium phosphate (e.g., HA-TCP, Zimmer Inc. Warsaw, IN) which is used for topical reconstruction of bones in the field of orthopedic surgery and dental field, may also be used as an appropriate carrier. Still further, in cases where flu  
20 like symptom such as fever is expected or actually occurs, to prevent or to relieve the symptom, an anti-inflammatory drug may also be used in combination with IFN without problem.

The accurate dose of the IFN according to the  
25 present invention varies depending on the age, body weight and sex of the patient; as well as on the type, characters and stage of the disorder. Therefore, the

accurate dose is not determined beforehand and determined by the physician. However, usually, appropriate dose for systemic administration per day per man is about 10,000 units to about 10,000,000 units, and appropriate dose for  
5 topical administration is usually about 10,000 units to about 3,000,000 units.

The present inventors studied the obtained experimental results to prove for the first time that IFN inducers which induce IFN $\alpha$ ,  $\beta$  or  $\gamma$  have the potential as  
10 drugs for prevention or treatment of bone disorders irrespective whether the inducer is a low molecular substance or a high molecular substance. Examples of the IFN inducer which may be used as a drug for treating bone disorder include commercially available drugs such as OK-  
15 432 (Saito, M. et al., Cell Immunol. 68, 187, 1982), PSK (Tsuru, S. et al., Cancer Immunol. Immunother., 22, 114, 1986) and Levamisole (Matsubara, S. et al., Cellular Immunol., 43, 214, 1979); various live viruses having low toxicities; inactivated viruses; culture media and  
20 treated cells of bacteria having low toxicities (e.g., Izumi, S. et al., Cancer Res., 46, 1960, 1986; H. Hara et al., Biotherapy 3, 1607, 1989; T. Takahashi et al., Report on the 44th Japan Cancer Association General Meeting, p.153, 1985; and Japanese Patent Nos. 1041679, 863592, 1287243, 988498, 1151765 and 1114570); naturally  
25 occurring or synthetic RNAs, derivatives thereof and complexes thereof (e.g., Japanese Patent Nos. 1139721,

877116 and 1113637, Japanese Laid-open Patent Application (Kokai) Nos. 2-111723 and 59-93097); plants and substances derived from plants, such as glycyrrhizin (e.g., I. Urushizaki and S. Tsukagoshi eds., "Cancer and BRM" pp.187-188, Science Forum, 1982; Japanese Patent Nos. 1281580, 1681643 and 1288021, Japanese Laid-open Patent Application (Kokai) No. 4-77431); organic germanium compounds (e.g., Japanese Laid-open Patent Application (Kokai) No. 7-247296; Japanese Patent Nos. 1661336, 1418821, Japanese Laid-open Patent Application (Kokai) No. 58-18395); straight chain lower saturated carboxylic acids such as butyric acid, fats and fatty acids (e.g., Japanese Laid-open Patent Application (Kokai) No. 7-31495, Japanese Patent No. 1701933 and Japanese Laid-open Patent Application (Kokai) No. 2-78623), peptides and proteins (e.g., Japanese Laid-open Patent Application (Kokai) Nos. 4-51892, 59-222422, 59-27832 and 58-15923); pyrimidinol compounds (e.g., Japanese Patent No. 1905169); polysaccharides (e.g., Japanese Patent Nos. 1643537, 1449562 and 1393449); dipyridamol compounds (e.g., Japanese Laid-open Patent Application (Kokai) No. 58-170715); carbamylpiperazine compounds (Japanese Patent No. 1290606), imiquimod (e.g., Sidky, Y. A. et al., Cancer Res., 52, 3528, 1992); and ray fungi and culture products thereof (e.g., Japanese Laid-open Patent Application (Kokai) No. 57-58630), although the examples are not restricted to these. Any substance which induces



production of IFN when administered to the body may be employed (S. Kobayashi, "Interferon", Supplemented Edition, Chapter 9, p.19, Kodansha Scientific, 1978).

Although the IFN inducers may be used as drugs as they  
5 are, they may be admixed with one or more  
pharmaceutically acceptable carrier or additive.

Examples of these include almost all of the carriers  
suited for parenteral administration, such as water,  
physiological saline, Ringer's solution, Hanks' solution  
10 and glucose solution, as well as solutions of lactose,  
dextrose, ethanol, glycerol, albumin and the like. These  
compositions may contain other additives such as  
stabilizers, antioxidants, antibacterial agents,  
antiseptics, buffer agents, surfactants and the like as  
15 required. The administration route is not restricted,  
and appropriately selected depending on the disorder to  
be treated. The drug may be administered orally;  
parenterally or systemically, that is, subcutaneously,  
intramuscularly, intraperitoneally, intravaginally,  
20 intrarectally, or by intravenous injection; and/or  
intranosally and/or intrapulmonally. Further, a  
sustained release system of indwelling type such as an  
osmotic pump (e.g., alzet osmotic pump) may be selected  
depending on the disorder to be treated. When the drug  
25 is administered intranosally or intrapulmonally, the drug  
may be in the form of solution or powder formulated by a  
medically acceptable device which produces fine solution

drops or fine powder.

Further, the IFN inducer according to the present invention may be formulated as one for topical application. For example, the drug may be directly  
5 infused to the site to be treated, or a carrier of the drug which allows for sustained release of the drug may be directly embedded in the site to be treated. Examples of such a carrier include hydrogels, polylactic acid and collagen matrices. In cases where a collagen matrix  
10 originated from an animal other than human is employed, it is preferred to use atelocollagen (such as Zyderm, Collagen Corp., Palo Alto CA) in which the antigenecity of collagen is deleted. Further, hydroxyapatite calcium phosphate (e.g., HA-TCP, Zimmer Inc. Warsaw, IN) which is  
15 used for topical reconstruction of bones in the field of orthopedic surgery and dental field, may also be used as an appropriate carrier.

Further, in cases where flu like symptom such as fever is expected or actually occurs, to prevent or to  
20 relieve the symptom, an anti-inflammatory drug may also be administered before, simultaneously, or after the administration of the IFN inducer without problem. The method of combinational administration and doses of the drugs are determined by the physician taking into  
25 consideration the age, body weight and sex of the patient, as well as the type, characters and stage of the disorder. Since it is known that IFN per se is pyrogenic,

it is very effective to incorporate an anti-inflammatory drug as an ingredient in the IFN inducer formulation.

#### Examples

5 The present invention will now be described by way of examples thereof. It should be noted, however, the examples are presented for the illustration purpose only and the present invention is not restricted to the examples.

#### Example 1

10 Action on Formation of Osteoclast Like Cells of Mice of ddy Strain

##### 1) Method for Preparing and Culturing Bone Marrow Cells

Bone marrow cells were prepared in accordance with the method by Takahashi et al (Takahashi, N. et al.,  
15 Endocrinology, 122, 1373, 1988). Male mice of ddy strain of 7 weeks old were exanguinated to death under ether anesthesia and the femur and the tibia of hind legs were collected from each mouse. The connective tissues on the surfaces of the bones were carefully removed, and both  
20 ends of each bone were cut off with scissors. The bone marrow cells were suspended in  $\alpha$ -Minimum Essential Medium ( $\alpha$ -MEM, Nissui Pharmaceuticals Co.) containing 10% precolostrum new born calf serum (Mitsubishi Chemical, hereinafter referred to as "JCS" for short) and the  
25 suspension was flushed out through a 25G needle attached to a syringe to collect the suspension in a centrifugal tube. The collected suspension was washed by

centrifugation in the same medium at 2000 rpm at 4°C for 5 minutes. The precipitated cells were dispersed in the above-mentioned medium and the washing by centrifugation was repeated twice, thereby preparing bone marrow cells.

5 Murine IFN- $\alpha$  (hereinafter referred to as "MuIFN $\alpha$ " for short), Lot. B13010,  $10^6$  units/ml/vial (Calbiochem-Novabiochem International, San Diego, CA), murine IFN- $\beta$  (hereinafter referred to as "MuIFN $\beta$ " for short), Lot M-0034,  $10^7$  units/ml/vial (Toray Industries, Inc.) and  
10 murine IFN $\gamma$  (hereinafter referred to as "MuIFN $\gamma$ " for short), Lot 254F3,  $2.06 \times 10^6$  units/ml (Genentech, CA) were respectively dissolved in 10% JCS-containing  $\alpha$ -MEM (Nissui Pharmaceuticals Co.) to the desired concentrations. Active vitamin D<sub>3</sub> (1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, Wako Pure Chemical Industries, Ltd.,  
15 hereinafter referred to as "Vit D<sub>3</sub>" for short) was dissolved in argon-saturated ethanol, and the obtained solution was diluted so as to attain the final ethanol concentration of 0.1% in all of the culture systems.

20 2) Evaluation of Ability to Form Osteoclasts

The number of viable bone marrow cells was counted using tripam blue dye exclusion test and the bone marrow cells were diluted with  $\alpha$ -MEM containing 10% JCS,  $10^{-8}$  M Vit D<sub>3</sub>,  $10^{-7}$  M dexamethasone to a population density of  
25  $1.5 \times 10^6$  cells/ml, followed by placing the resulting suspension in each well in 24-well plates in an amount of 0.45 ml/well. To each well, 50  $\mu$ l of the medium

containing varying concentration of IFN was added to make the volume to 0.5 ml/well and culturing was begun. At every three days, 0.4 ml of the culture medium was removed from each well and 0.4 ml of fresh medium (the above-mentioned medium containing the respective concentration of IFN) was added. On Day 8 from the beginning of the culturing, the medium was removed and the cells were fixed with mixed solution of ethyl alcohol and diethyl ether (1:1) and the cells were stained with tartrate resistant acid phosphatase (TRAP), followed by counting TRAP positive multinuclear cells with a phase contrast microscope. Fig. 1 shows the results. Both the MuIFN $\beta$  and MuIFN $\gamma$  added to each of the above-mentioned system strongly inhibited formation of osteoclast like cells. Each column in the figure shows the average and standard error of 4 cases. Clear dose dependence was observed in both IFNs. The inhibition effect of IFN $\gamma$  was stronger than that of IFN $\beta$ . That is, the inhibition concentration IC<sub>50</sub> of IFN $\gamma$  was not more than 0.1 units/ml, and that of IFN $\beta$  was 0.2 units/ml.

#### Example 2

#### Action on Formation of Osteoclast Like Cells of Mice of C 57 BL/6 Strain

In place of mice of ddy strain, male mice of C 57 BL/6 of 7 weeks old were used. The mice were exanguinated to death under ether anesthesia and the femur and the tibia of hind legs were collected from each

mouse. The connective tissues on the surfaces of the bones were carefully removed, and both ends of each bone were cut off with scissors. The bone marrow cells were suspended in  $\alpha$ -MEM containing 10% JCS and the suspension was flushed out through a 25G needle attached to a syringe to collect the suspension in a centrifugal tube. The collected suspension was washed by centrifugation in the same medium at 2000 rpm at 4°C for 5 minutes. The precipitated cells were dispersed in the above-mentioned medium and the washing by centrifugation was repeated twice. Formation of osteoclast like cells was measured by the same method as in Example 1. As a result, as in the case of mice of ddy strain, both  $\text{MuIFN}\beta$  and  $\text{MuIFN}\gamma$  strongly inhibited formation of osteoclast like cells from bone marrow cells of mice of C 57 BL/6 strain. Fig. 2 shows the results. The  $\text{IC}_{50}$  values of formation inhibition of osteoclast like cells of  $\text{MuIFN}\beta$  and  $\text{MuIFN}\gamma$  were 0.1 units/ml and 1.5 units/ml, respectively. Each column in the figure shows the average and standard error of 4 cases.

### Example 3

Action on Proliferation of Murine Osteoblasts (*in vitro* single administration)

Murine osteoblast like cells MC3T3-E1 originated from C 57 BL/6 mouse (Kodama et al., Jap. J. Oral Biol., 23, 899, 1982) were washed with PBS(-) and the cells were dissociated by incubation in PBS(-) containing 0.1%

pronase (Actinase E, Kaken Pharmaceuticals Co.), followed by dispersing the cells in  $\alpha$ -MEM containing 10% JCS and 50  $\mu$ g/ml of L-ascorbic acid (ASA). The ability to promote proliferation of cells was evaluated as follows.

5 That is, MC3T3-E1 cells were placed in each well in 24-well plates (Corning) in an amount of 5000 cells/well. Then each IFN dilution was added to each well in an amount of 0.1 ml/well, and the culturing was begun. On Day 2, the medium was removed and the each IFN dilution  
10 was added in an amount of 0.5 ml/well (total amount of liquid: 0.5 ml/well). On Day 6, the medium was removed and the cells were washed with PBS(-) and a mixed enzyme solution of 0.1% pronase/0.1% collagenase was added to each well in an amount of 200  $\mu$ l/well, followed by  
15 allowing to react at 37°C for 15 minutes. Then  $\alpha$ -MEM containing 10% fetal calf serum (Gibco, hereinafter referred to as "FCS" for short) was added in an amount of 300  $\mu$ l/well to disperse the cells therein and the number of the cells was counted with a Coulter counter.

20 The actions on cell proliferation are shown in Fig. 3. Each column in the figure shows the average and standard error of 4 cases. Both murine IFNs showed very weak or substantially no inhibition actions on the proliferation of MC3T3-E1 cells. Although slight  
25 inhibition of proliferation was observed with MuIFN $\gamma$  from the dose of 1 unit/ml, the dose dependence is small, and the inhibition was only about 30% even at a concentration

as high as 10,000 units/ml. On the other hand, MuIFN $\beta$  did not inhibit the proliferation at all even at 10,000 units/ml.

#### Example 4

#### 5 Actions on Differentiation Ability and Mineralization of Murine Osteoblasts (*in vitro* single administration)

As the parameters for evaluation of differentiation ability, alkaline phosphatase (ALPase) and mineralization were employed. Detection of alkaline phosphatase which is a marker of differentiation of osteoblasts was carried out on Day 8 from the beginning of the culturing by staining method. Evaluation of mineralization was carried out as follows. That is, MC3T3-E1 cells were placed in each well of 24-well plates in an amount of 15 50,000 cells/ml (10 times in the case of anti-cell test in Example 3). On Day 4, after confirming that the cells sufficiently reached confluency, the medium was discarded and fresh  $\alpha$ -MEM containing 10% JCS, 50  $\mu$ g/ml of L-ascorbic acid and 10 mM  $\beta$ -glycerophosphate was added in 20 an amount of 0.9 ml/well, followed by adding medium containing varying concentration of IFN. Four days later, the medium was replaced with IFN-free medium, and the medium was replaced with IFN-free medium every 4 days. On Day 14, Von Kossa stain was performed to visualize the 25 calcified foci. Expression of alkaline phosphatase activity which is a differentiation marker is an indispensable step to the subsequent mineralization.



Both murine IFNs did not inhibit expression of the enzyme activity which is a differentiation marker.

On the other hand, a large difference was observed between MuIFN $\beta$  and MuIFN $\gamma$  in the mineralization process as shown in Fig. 4. That is, while mineralization proceeded without substantial influence even in the presence of IFN $\beta$ , IFN $\gamma$  showed strong and dose dependent inhibition action on mineralization. The inhibition effect was expressed at a concentration as low as 10 units/ml, and IC<sub>50</sub> value of inhibition of mineralization was about 3-4 units/ml. Needless to say, for complete repair of the bone tissues as hard tissues, the inhibition of mineralization by MuIFN $\gamma$  is not preferred, so that the superiority of IFN $\beta$  to IFN $\gamma$  is apparent.

#### Example 5

Quantification of Calcium and Inorganic Phosphorus in Calcified Foci of Murine Osteoblasts MC3T3-E1

MC3T3-E1 cells were placed in each well in 24-well plates in an amount of 50,000 cells/well and cultured to confluency. The medium was removed and fresh  $\alpha$ -MEM containing 10% JCS, 50  $\mu$ g/ml of L-ascorbic acid and 10 mM  $\beta$ -glycerophosphate was added in an amount of 0.9 ml/well, followed by adding 0.1 ml/well of medium containing varying concentration of IFN. The medium was replaced with IFN-free medium every 4 days. On Day 14, the medium was removed and the cells were lightly washed with Hank's solution. Anhydrous ethanol was added in an amount of 1

ml/well and fixation was carried out at room temperature for 15 minutes. Then the anhydrous ethanol was replaced with fresh anhydrous ethanol in an amount of 1 ml/well and 5 minutes later, each well was dried in the air.

5 Then 1N hydrochloric acid was added in an amount of 1 ml/well and extraction was carried out at room temperature for 30 minutes. The total contents of each well were transferred to an Eppendorf tube and centrifuged at 1800 x g at 4°C for 10 minutes. The  
10 amount of calcium in the supernatant was measured by Calcium C Test Wako<sup>®</sup> and the amount of inorganic phosphorus in the supernatant was measured by Phospha B Test Wako<sup>®</sup>. Although slight inhibition of calcium  
15 deposition was observed with the addition of IFN $\beta$ , the degree was small and the calcium deposition was only 20% smaller than the control even at a concentration of 1000 units/ml. On the other hand, IFN $\gamma$  exhibited dose dependent and strong inhibition of mineralization and the  
20 calcium deposition was as much as 70% smaller than the control at a concentration of 1000 units/ml. The IC<sub>50</sub> value was as low as about 10 units/ml (Fig. 5). Each point in the figure shows the average and standard error of 4 cases.

The amounts of inorganic phosphorus extracted from  
25 calcified foci are shown in Fig. 6. Each point in the figure shows the average of 4 cases. The amounts of the extracted inorganic phosphorus showed good correlation

with the amounts of calcium. The amount of the extracted inorganic phosphorus for 1000 units/ml of IFN $\beta$  was only about 20% smaller than the control and IC<sub>50</sub> value could not be obtained. On the other hand, the amount of the extracted inorganic phosphorus for 1000 units/ml of IFN $\beta$  was about 80% smaller than the control and the IC<sub>50</sub> value was about 10 units/ml, which is extremely small (Fig. 6).

#### Example 6

Actions on Differentiation Ability and Mineralization of Murine Osteoblasts (*in vitro* multiple administration)

MC3T3-E1 cells were placed in each well in 24-well plates in an amount of 30,000 cells/well and cultured to confluency. The medium was removed and fresh  $\alpha$ -MEM containing 10% JCS, 50  $\mu$ g/ml of L-ascorbic acid and 10 mM  $\beta$ -glycerophosphate was added in an amount of 0.9 ml/well, followed by adding 0.1 ml/well of medium containing varying concentration of IFN. The medium was replaced with the medium containing the respective concentration of IFN every 4 days. On Day 9 from the first treatment with IFN, Von Kossa stain was performed to visualize the calcified foci. As a result, unlike the case of single administration, by the twice administration of IFN, the number of calcified foci and the intensity of staining were dose dependently increased (Fig. 7).

#### Example 7

Therapeutic Effect of Murine IFN $\beta$  Against Osteoporosis in Model Mice

Female mice of ddy strain of 8 weeks old were divided into 5 groups and experiment was carried out. Bilateral ovariectomy was performed on the mice in 3 groups and sham operation alone was performed on the mice in 2 groups. Thereafter, the mice were raised with normal diet for 1 month, thereby making the state of osteoporosis in the ovariectomized groups. In this model, it is known that the mice become the state of osteoporosis 2 to 3 weeks after the ovariectomy (T. Hosaka et al., ANITEX, Vol. 5, 243, 1992; Miyaura, C. et al., J. Bone Mineral Res., 10, 1365, 1995). From the third weeks from the ovariectomy, murine IFN was subcutaneously administered (100 µl/head/time) once per day, totally 25 times. On Day 30 from the beginning of administration, under ether anesthesia, body weight was measured, blood was collected, and femur was collected and dry bone weight thereof was measured for each mouse.

Drying of femur was performed as follows. That is, femur was fixed with 2 ml of 70% ethanol per one taken out femur at room temperature for 24 hours and then the attached muscles and connective tissues were carefully removed with a forceps and surgical knife with replaceable blade. After immersing the femur in fresh 70% ethanol for 72 hours at room temperature, the femur was dried in a hot wind-generating incubator (Koukensha Engineering Co., Tokyo) at 55°C for 24 hours. The weight of the dried bone was measured with an electronic force

balance (Mettler AE50, Metteler Instrumente AG, Switzerland). Murine IFN to be administered was diluted to the desired concentration with physiological saline. During the test, urine of mice was collected with metabolic cage (KN-645, Natsumeseisakusho, Tokyo) and frozen to store. To one group subjected to the sham operation, physiological saline (Otsuka Pharmaceutical Co.) was administered, and to another group subjected to the sham operation, murine IFN $\beta$  was administered at a dose of  $10^5$  units/head/time (diluted with physiological saline when use). To one group subjected to ovariectomy, physiological saline was administered and to the other two groups subjected to ovariectomy, MuIFN $\beta$  was administered at doses of  $10^3$  units/head/time and  $10^5$  units/head/time, respectively. Fig. 8 shows the dry bone weights. Ovariectomy which is a model of post menopausal osteoporosis caused clear decrease in bone volume in mice. However, administration of MuIFN $\beta$  at a dose of  $10^5$  units/head/time recovered the normal level of bone volume, so that MuIFN $\beta$  exhibited clear therapeutic effect. On the other hand, in the groups subjected to the sham operation, significant increase in the bone volume was not observed even at a dose of  $10^5$  units.

It is well-known that the increase and decrease in the amount of urinary D-Pyr excreted in urine, which is a crosslinked substance of bone collagen, is an excellent marker reflecting the degree of degradation of bone

collagen, that is, the degree of bone resorption (C.P. Jerome et al., Bone and Mineral, 19, 117-125, 1992). For measurement of urinary D-Pyr, a commercially available kit (PYRILINKS - D Assay, METRA BIOSYSTEMS) was used.

5 The amount of D-Pyr of mice in each group compensated with the amount of the urinary creatinine in Week 4 from administration of MuIFN $\beta$  is shown in Fig. 9. In the group to which physiological saline was administered, the amount of D-Pyr which is a bone resorption marker clearly  
10 increased, while in the groups to which MuIFN $\beta$  was administered at doses of  $10^3$  units/head/time and  $10^5$  units/head/time, respectively, the amount of excreted D-Pyr was smaller, that is, inhibition of bone resorption was observed. The degree of inhibition was about the  
15 same level as in the group subjected to sham operation and administered with physiological saline, that is, about the same level as the normal value in urine. Fig. 10 shows the change in body weight before and after beginning of administration of MuIFN $\beta$ . Although not  
20 shown in the figure, the body weight of the mice immediately before the ovariectomy (3 weeks before the commencement of administration of MuIFN $\beta$ ) was  $25.6 \pm 0.18$  g (n=33) and that of the group to be subjected to sham operation was  $25.7 \pm 0.36$  g (n=11), so that the body  
25 weights of the groups were about the same. Although at 2 weeks after the surgery, that is, at 1 week before commencement of administration of IFN, the body weights

of the groups were different, this reflects the increase in body weight due to ovariectomy (Miyaura, C. et al., J. Bone Mineral Res., 10, 1365, 1995). By autopsy after the test, large decrease in uterine weight in the

5 ovariectomized group was confirmed. This is also a typical response due to ovariectomy (Miyaura, C. et al., J. Bone Mineral Res., 10, 1365, 1995). Administration of IFN $\beta$  continuously for 4 weeks at a dose exhibiting the therapeutic effect, that is, at a dose at which the bone  
10 volume is increased, no problematic change in body weight was observed in any group.

#### Example 8

#### Comparison of Effects of Various Murine IFNs on Inhibition of Formation of Murine Osteoclasts

15 The effects of the three murine IFNs, that is, MuIFN $\beta$ ,  $\gamma$  and  $\alpha$  in terms of abilities to inhibit formation of osteoclast like cells from bone marrow cells in mice of ddy strain were compared. The test was carried out as in Example 1. All of the murine IFNs  
20 inhibited the formation of osteoclast like cells, and the effect was strong in the order of  $\gamma$ ,  $\beta$  and  $\alpha$ . The IC<sub>50</sub> values determined from the dose dependence curves thereof were not more than 0.1 unit/ml for IFN $\gamma$  and about 0.9 units/ml for IFN $\beta$  while that for IFN $\alpha$  was 22.4 units/ml.  
25 Thus, the activity of IFN $\alpha$  to inhibit formation of osteoclasts was about 1/20 of that of IFN $\beta$  and about not more than 1/100 of that of IFN $\gamma$ .

## INDUSTRIAL AVAILABILITY

As described above, by the present invention, the relative action to promote bone formation by IFN $\beta$  was proved in *in vitro* tests and in animal tests, so that the usefulness of IFN $\beta$  as a drug for treating and preventing bone disorders was shown. Especially, the discovery that the recovery of the bone volume by IFN $\beta$  *in vivo* is more prominent in the individuals having abnormal bone volumes than in the individuals having normal bone volumes provides a high advantage to the industrial use of IFN $\beta$  as a drug for treating bone disorders.

Further, the possibility to use interferon inducers as drugs for treating bone disorders was discovered, which provides a high advantage to the industrial use of IFN $\beta$  as a drug for treating bone disorders.



## CLAIMS

1. A drug for treating bone disorders comprising as an effective component interferon  $\beta$  or an interferon inducer.
2. The drug according to claim 1, wherein said  
5 interferon  $\beta$  is a naturally occurring or a recombinant interferon  $\beta$ .
3. The drug according to claim 1, wherein the interferon produced by said interferon inducer is interferon  $\alpha$ ,  $\beta$  or  $\gamma$ .
- 10 4. The drug according to any one of claims 1 - 3, wherein said bone disorder is osteoporosis, a tumor-related disorder, metabolic disorder, periodontal disease-related disorder or bone fracture.
5. A pharmaceutical composition for treating bone  
15 disorders comprising interferon  $\beta$  or an interferon inducer and a pharmaceutically acceptable carrier or diluent.
6. Use of interferon  $\beta$  or an interferon inducer as a drug for treating bone disorders.
- 20 7. A method for treating bone disorders comprising using interferon  $\beta$  or an interferon inducer.
8. A method for searching and evaluating a substance effective for prevention or therapy of bone disorders comprising employing ability to induce interferon  
25 production as an index.

## ABSTRACT

By the present invention, the relative action to promote bone formation by IFN $\beta$  was proved. The action to inhibit mineralization as shown by interferon  $\gamma$  is not observed with interferon  $\beta$ . Especially, usefulness of interferon  $\beta$  as a drug for treating bone disorders was shown, whose effect for prevention and therapy of bone disorders was confirmed in the bone disorder model animals in terms of increase in the bone volume.

Further, the possibility of using interferon inducers as drugs for treating bone disorders was discovered.

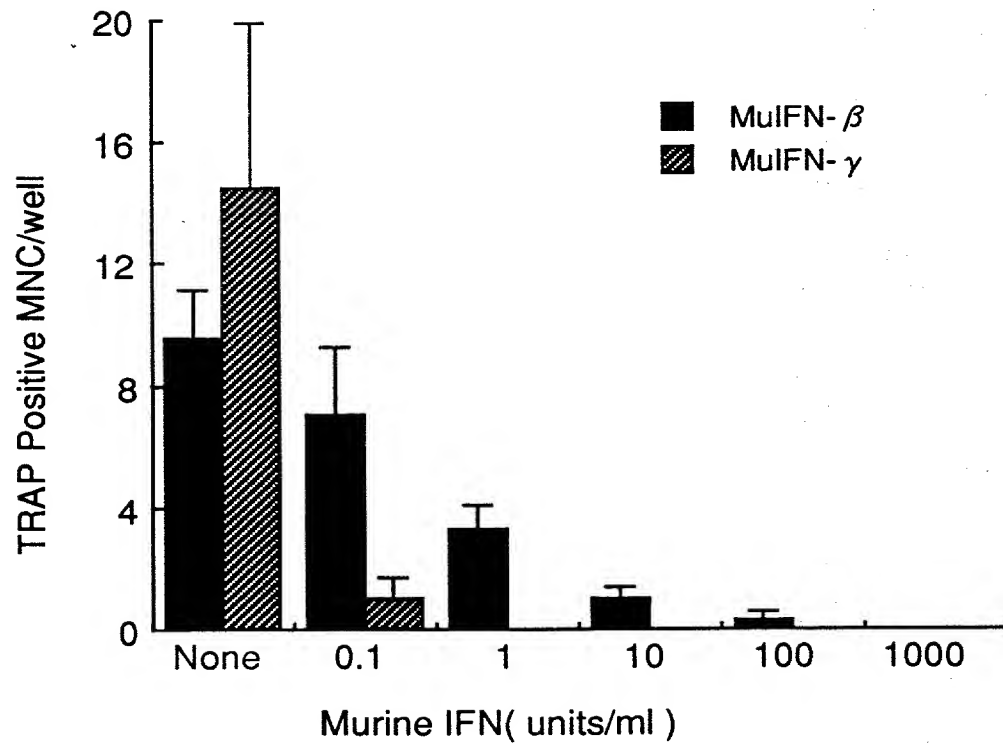


Fig.1 : Inhibition Effect of Murine IFN on Formation of TRAP positive multinucleated Cells in in vitro Bone Marrow Cell Culture. Bone Marrow Cells were prepared from ddY mice. MNC ;Multinucleated Cells, TRAP; Tartrate Resistant Acid Phosphatase. Mean  $\pm$  S.E.( n=4).

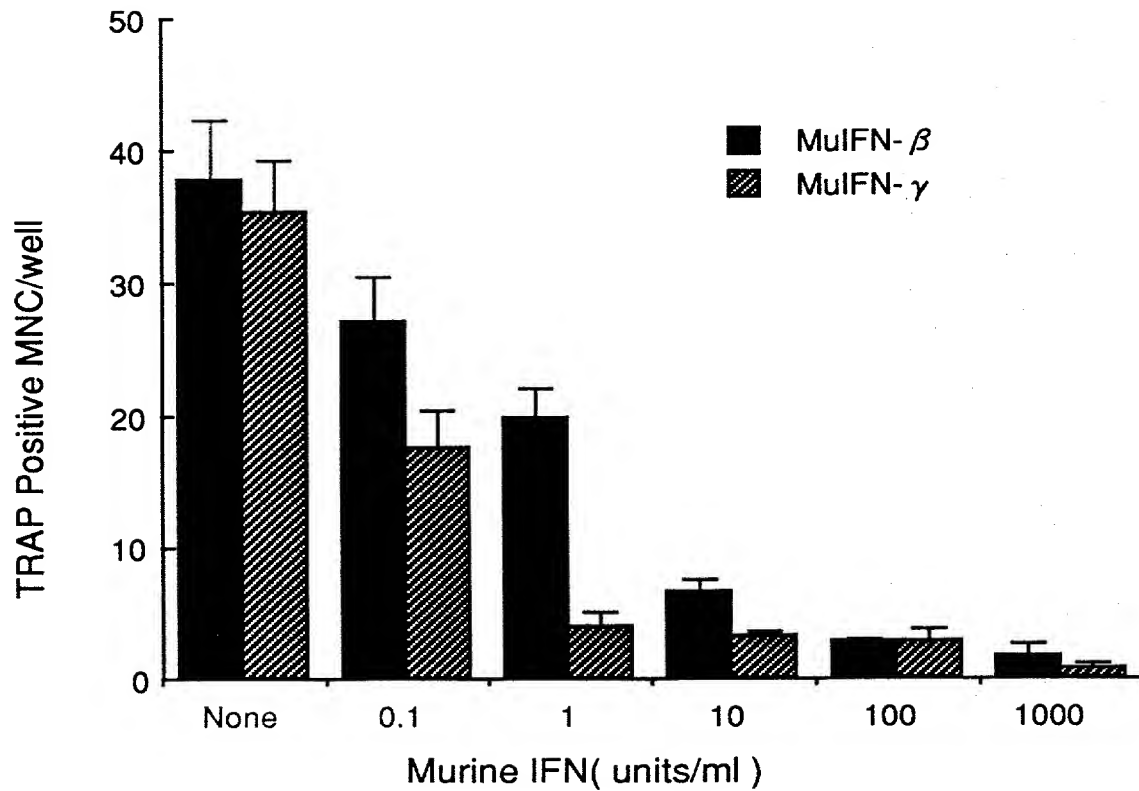


Fig.2 : Inhibition Effect of Murine IFN on Formation of TRAP positive multinucleated Cells in in vitro Bone Marrow Cell Culture. Bone Marrow Cells were prepared from C57BL/6 mice. MNC ;Multinucleated Cells, TRAP; Tartrate Resistant Acid Phosphatase. Mean  $\pm$  S.E.( n=4).

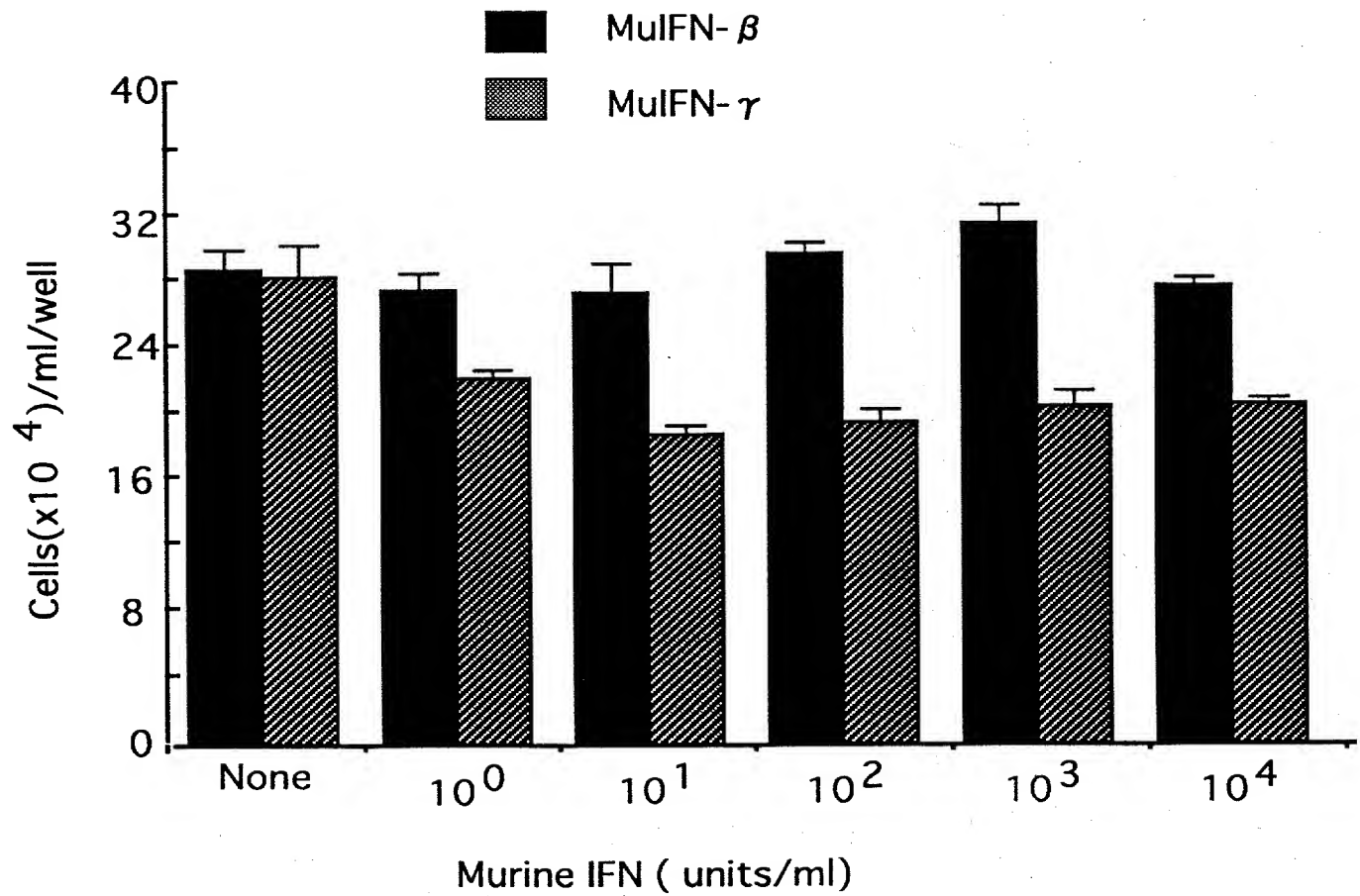
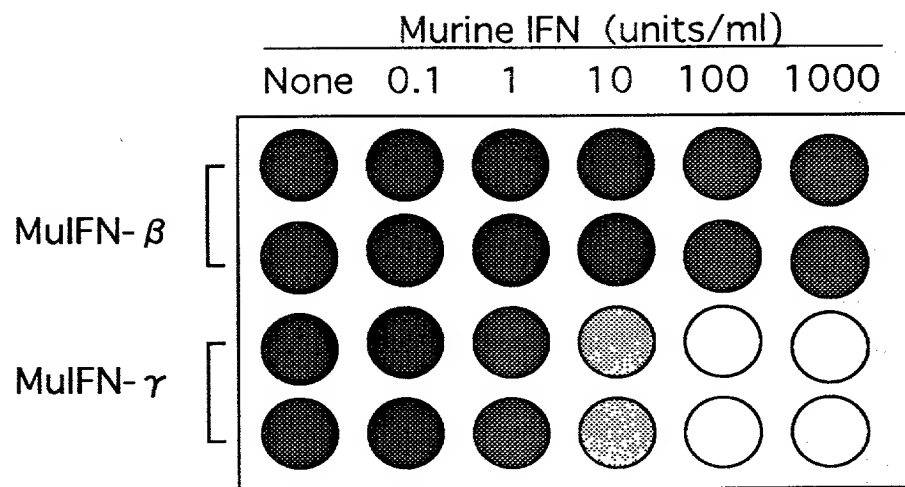


Fig.3 : Effect of Murine IFN on Proliferation of Murine Osteoblast Like Cells MC3T3-E1. Mean  $\pm$  S.E.(n=4).



**Fig.4 : Effect of Murine IFN on Mineralization of Murine Osteoblast Like MC3T3-E1 Cells in Culture. Mineralization was visualized by Von Kossa Staining.**

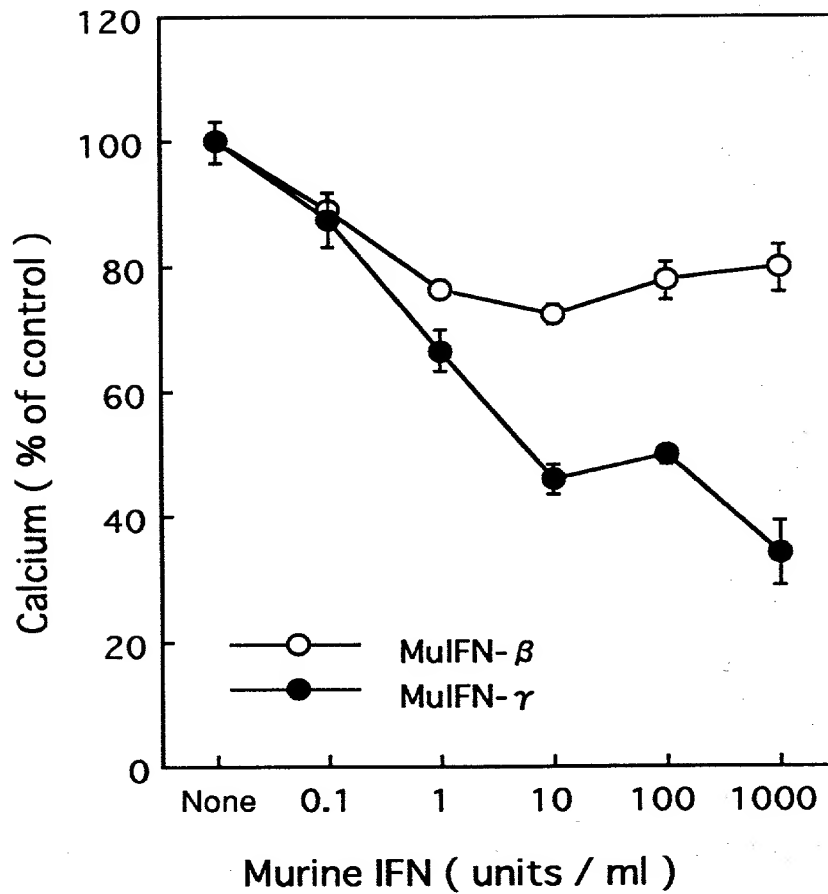


Fig.5 : Amount of Calcium Deposited on Calcified Foci in the Culture of MC3T3-E1 Cells .Each Point Represents Mean  $\pm$  SE.

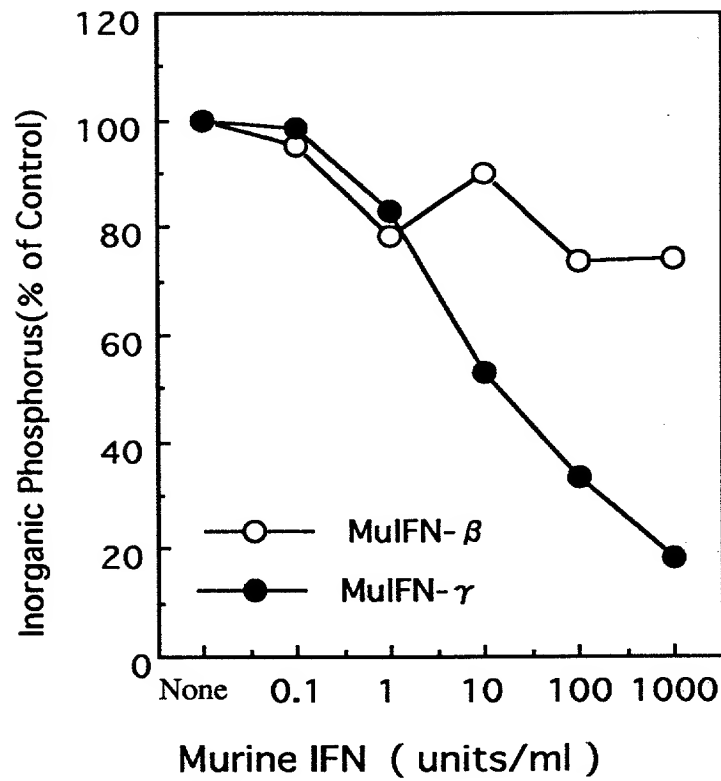


Fig.6 : Amount of Inorganic Phosphorus Deposited on Calcified Foci in the Culture of Murine Osteoblast Like Cells MC3T3-E1.



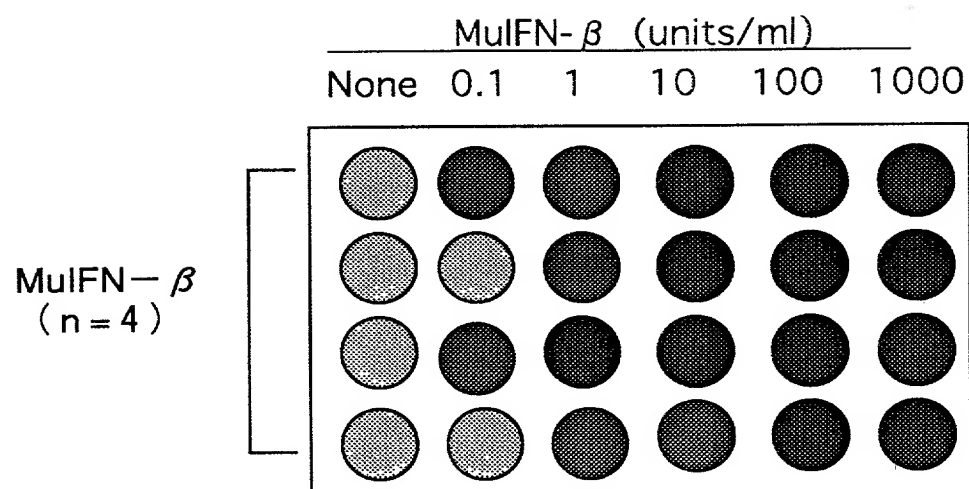


Fig.7 : Effect of Multiple Administration of Murine interferon  $\beta$  to Promote Mineralization of Murine Osteoblast Like Cells MC3T3-E1.

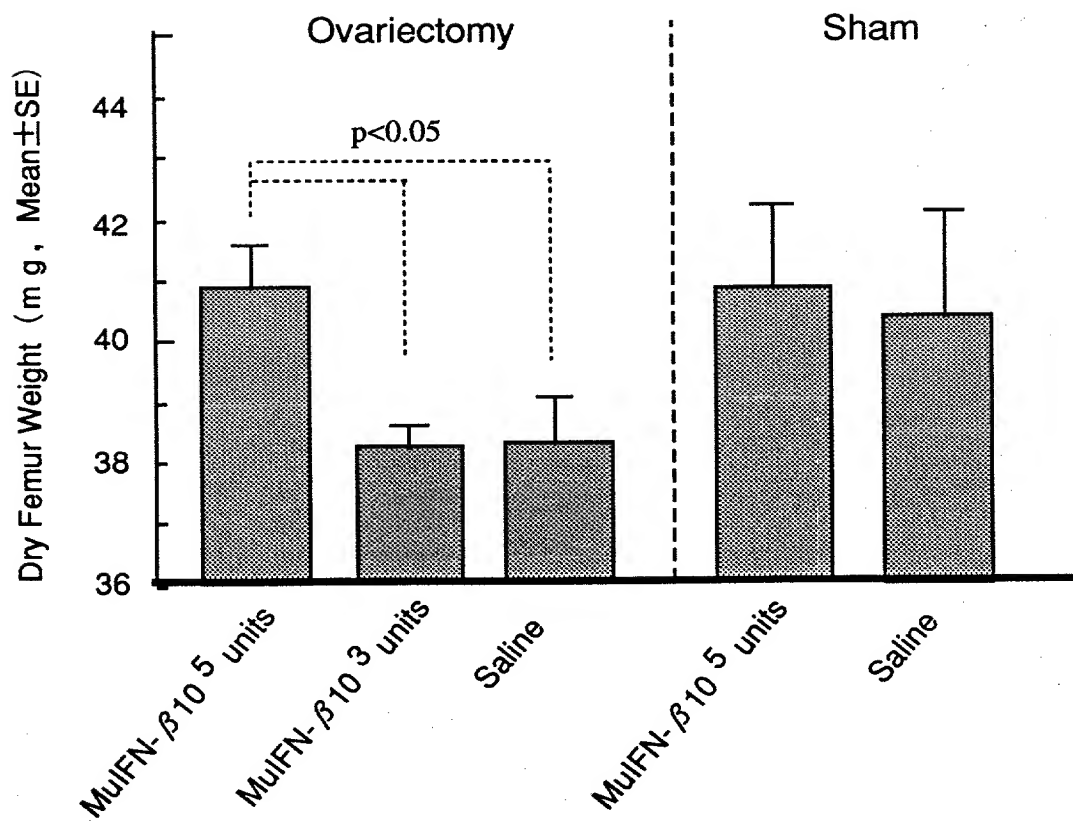


Fig.8 : Therapeutic Effect of Murine Interferon  $\beta$  on Post Menopausal Osteoporosis Model Mice.

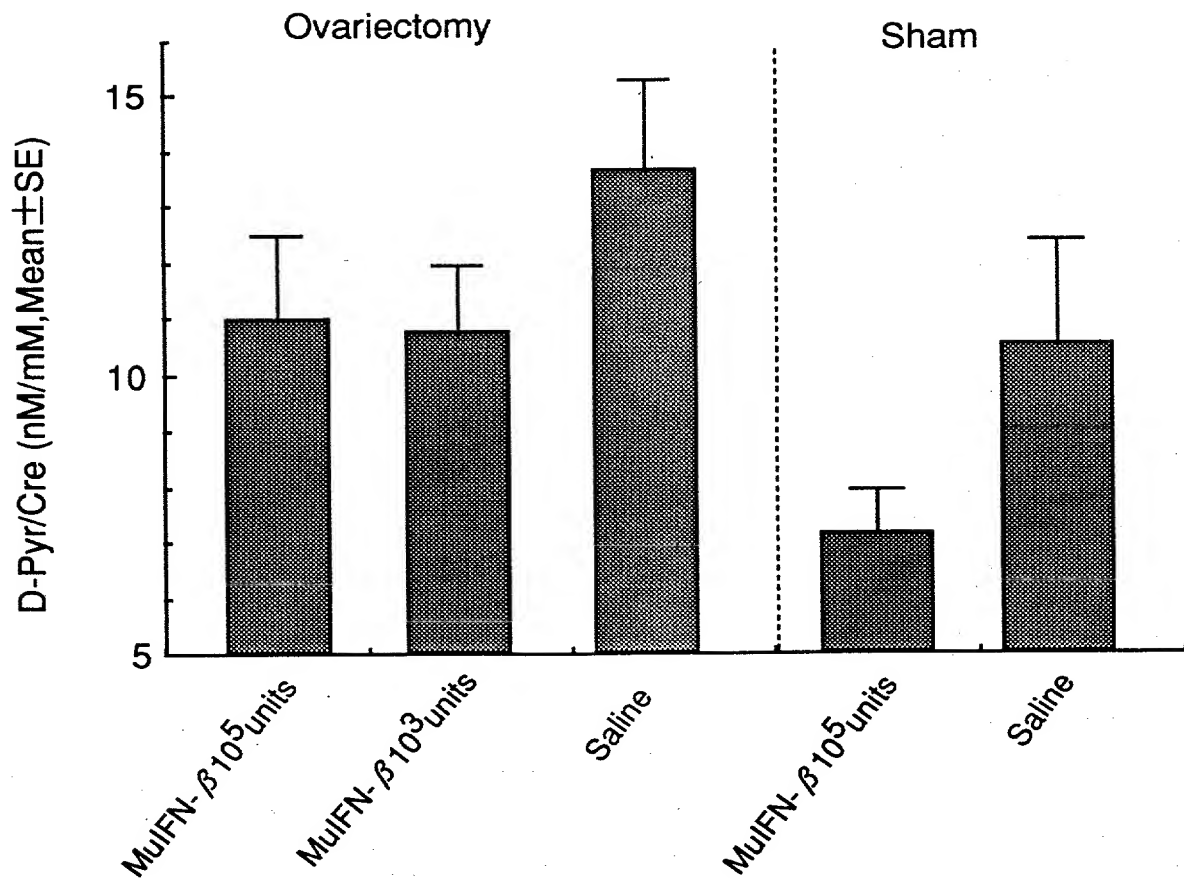


Fig.9 : Inhibiting Effect of Murine Interferon  $\beta$  on Degradation of Bone Collagen in Post Menopausal Osteoporosis Model Mice. D-Pyr; Deoxypyridinoline, Cre; Creatinine



**Fig.10: Change in Body Weight of Mice During Administration of Murine interferon  $\beta$  to Post Menopausal Osteoporosis Model Mice.**

# BIRCH, STEWART, KOLASCH & BIRCH, LLP

## COMBINED DECLARATION AND POWER OF ATTORNEY

### FOR PATENT AND DESIGN APPLICATIONS

ATTORNEY DOCKET NO.

599-158P

PLEASE NOTE:  
YOU MUST  
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FOLLOWING:

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:\*

Insert Title **REMEDY FOR BONE DISORDERS**

Check Box If  
Appropriate -  
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Specification  
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the specification of which is attached hereto unless the following box is checked:

☐ was filed on July 25, 1996 as United States Application Number \_\_\_\_\_ or PCT International Application Number PCT/JP96/02099 and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof, or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

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Information  
(if appropriate)

Prior Foreign Application(s)

Priority Claimed

<u>7/188972</u> (Number)	<u>Japan</u> (Country)	<u>07/25/95</u> (Month/Day/Year Filed)	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> No <input type="checkbox"/> No
<u>                    </u> (Number)	<u>                    </u> (Country)	<u>                    </u> (Month/Day/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> No <input type="checkbox"/> No
<u>                    </u> (Number)	<u>                    </u> (Country)	<u>                    </u> (Month/Day/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> No <input type="checkbox"/> No
<u>                    </u> (Number)	<u>                    </u> (Country)	<u>                    </u> (Month/Day/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> No <input type="checkbox"/> No
<u>                    </u> (Number)	<u>                    </u> (Country)	<u>                    </u> (Month/Day/Year Filed)	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> No <input type="checkbox"/> No

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(Application Number)

(Filing Date)

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(Application Number)

(Filing Date)

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\*NOTE: Must be completed.

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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 Inventor, if any:

see above

Full Name of Third  
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Full Name of Fourth  
 Inventor, if any:

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Full Name of Fifth  
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